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Extracellular Glycoprotein from Virulent and Avirulent *Cryptococcus* Species

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Two virulent strains of *Cryptococcus neoformans* and two nonvirulent forms (*C. albidus* and *C. laurentii*) were grown in liquid culture to produce maximal capsule formation. A glycoprotein was isolated from the culture medium and was homogeneous as determined by cellulose acetate electrophoresis and anion-exchange chromatography. The amino acid, neutral sugar, amino sugar, uronic acid, and *O*-acetyl compositions and the infrared spectra of the glycoprotein were determined. The product of the *C. neoformans* strains contained more mannose and uronic acid than did that from the nonpathogenic strains. *O*-acetyl groups were absent from glycoprotein of the two nonpathogens.

The main capsular polysaccharide produced by species of *Cryptococcus* is reported to consist of a mannose backbone with side chains of xylose and glucuronic acid (5, 6, 33) in varying proportions. Infrared spectroscopy has shown that differences in serological types depend in part on the degree of acetylation of polysaccharide side chains (29). Soluble polysaccharides from virulent strains are larger and contain more uronic acid residues than weakly virulent strains (24). The protein content of extracellular material has not been investigated, although yeasts are known to produce external enzymes (27). Quantities of individual extracellular polysaccharides are often limited, and in such cases appropriate microanalytical procedures are needed (9).

Growth conditions affect the production and composition of cryptococcal extracellular and capsular material (8, 14, 15, 30, 42), and media have been developed which enhance capsule production (30).

We report here results of experiments in which virulent and nonvirulent species of *Cryptococcus* were grown in culture with maximal capsule production. An extracellular glycoprotein was isolated and characterized by microanalytical procedures. The glycoprotein from *C. neoformans* contained *O*-acetyl groups and more mannose and uronic acid than did the molecule from the nonpathogenic species, which did not contain *O*-acetyl groups.

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MATERIALS AND METHODS

Yeast isolates and culture conditions. *C. neoformans* strains 365-11 and 365-16 and *C. laurentii* 371-1 were obtained from L. Kapica, Department of Microbiology, McGill University, Montreal, Quebec, Canada. *C. albidus* H1354, isolated from human cerebrospinal fluid, was obtained from H. G. Weiser, Aarau, Switzerland. H. J. Phaff (personal communication) confirmed identifications by expanded assimilation tests. The *C. albidus* strain was identified as a typical representative of *C. albidus* var. *diffluens*.

All cultures were maintained at 4°C on Sabouraud dextrose (4%) agar slopes and were subcultured monthly. Inoculum cultures of each strain were prepared in 50 ml of Littman's capsule medium (LCM) (30) containing 0.5 g of dextrose which was added after autoclaving. Thiamine (10 μg/ml) was added before autoclaving. The cultures were shaken at 100 rpm in an R77 Metabolyte shaking water bath (New Brunswick Scientific Co., New Brunswick, N.J.) at 25°C until each strain reached exponential phase. Samples (5 ml) of exponential-phase cultures were transferred to 350 ml of the same medium in 1-liter Erlenmeyer flasks and were shaken at 90 rpm at 25°C. The cultures were grown until they reached stationary phase, as judged by absorbance measurements and pH (approaching 3.0). The pH of cultures containing pathogenic strains was adjusted to 6.0 with 0.1 N NaOH, and the cells were killed by heating the flasks in a water bath at 55°C for 90 min. All cells were harvested by centrifugation at 16,000 × *g* and washed twice with water to dislodge as much adhering capsular material as possible. Washings were combined with the culture fluid and passed through a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.) to remove whole cells and cell fragments.

Virulence testing with mice. Virulence was tested by intracerebral inoculation of 12 Swiss white mice per strain with a 10^6 cell suspension in sterile saline (0.02 to 0.04 ml). Deaths began to occur after 4 weeks. Mortality was expressed as time taken to kill 50% of the mice. Autopsies were performed, agar slants were inoculated with brain tissue, and smears were examined with fungicidal India ink by phase-contrast microscopy.

Preparation of extracellular material. The extraction of soluble polysaccharide complexes from the filtered medium was by the modified procedure of Farhi et al. (14). The culture fluids were concentrated to 1/10 volume by rotary evaporation at 50°C. Two volumes of absolute ethanol were added to the concentrate, and the solution was kept for 48 h at 4°C. The precipitate was collected by centrifugation at 300 × *g*. One volume of absolute ethanol was added to the supernatant, and the solution was kept for 48 h at 4°C. The precipitate was collected by centrifugation at 300 × *g*. The two precipitates were combined, washed twice with absolute ethanol, centrifuged at 300 × *g*, and suspended in deionized water. The suspension was dialyzed at 4°C against three changes of deionized water in a 20-liter Multiple Dialyzer (Oxford Laboratories, Foster City, Calif.). The nondiffusible material was cleared by centrifugation at 27,000 × *g* for 60 min at 2°C, dispensed into preweighed plastic beakers, and lyophilized. The beakers were reweighed, and the white fluffy material was stored in screw-capped vials at 4°C. At least two preparations were made from each strain grown under identical conditions. All analytical procedures were performed on alcohol-precipitated material.

Electrophoretic procedures. Polyacrylamide disc electrophoresis was performed according to the modified method of Ornstein and Davis (37; D. J. Fox, D. A. Thurman, and D. Boulter, Biochem. J. **87**:29P, 1963). The stacking gel was omitted from the columns. Samples (0.05 to 0.2 ml containing 15 to 20 mg/ml) were run at 3 mA per tube until the bromophenol blue marker reached the bottom of the gel column. Protein was detected by staining with 1% amido Schwarz in 7% (wt/vol) acetic acid for 1 h. Destaining was done for 1 to 3 days in 7% acetic acid. Carbohydrate was detected by a modified periodic acid-Schiff stain (38).

Cellulose acetate electrophoresis was performed in a Gelman electrophoresis unit (Gelman Sciences, Inc., Ann Arbor, Mich.). Extracellular material (2 mg) was dissolved in 500 μl of 0.05 M tris(hydroxymethyl)-aminomethane barbital-sodium barbital buffer, pH 8.8 (Gelman High Resolution Barbital Buffer). Samples (5 μl) were applied to the cellulose acetate strips (2.5 by 15 cm) and run at 3 mA per strip for 40 min. The strips were cut in half longitudinally. One half was stained with Alcian blue to detect glycoprotein (47); the other was stained for carboxyl groups with 1% Alcian blue in 2.5% (wt/vol) acetic acid and destained in two changes of 0.1 M citrate buffer, pH 3.0–absolute ethanol (1:1, vol/vol) and then cleared with two changes of absolute methanol (1 min each) and one rinse of 10% (vol/vol) acetic acid in absolute methanol (1 min). Separate strips were stained for protein with 0.25% Coomassie brilliant blue in 7% (wt/vol) acetic acid for 15 min (11). These strips were destained in four changes of 5% acetic acid and cleared as described above. The strips were dried at 60°C for 15 min on a grease-free glass plate. Heparin (for Alcian blue) and Hyland control serum (for Coomassie brilliant blue) were used as reference markers.

Gel chromatography. A 15-mg sample was suspended in 10 ml of 0.02 M pyridine hydrochloride buffer, pH 5.5, and then degassed. The solution was dialyzed at 4°C against three changes of the same buffer and was chromatographed on a column (37 by 2.5 cm) of diethylaminoethyl (DEAE)-Bio-Gel A (100 to 200 mesh; Bio-Rad Laboratories, Richmond, Calif.) which was equilibrated with the buffer. The column was eluted with 400 ml of the buffer followed by a convex gradient from 0 to 3.0 M NaCl. (The mixing chamber contained 700 ml of buffer, and the reservoir contained 700 ml of 3.0 M NaCl.) Fractions (10 ml) were collected, and portions (2 ml) were assayed for carbohydrate (10) and for protein by ultraviolet light absorbance at 280 nm. Conductivity was measured by using a conductivity bridge model RC216B2 (Industrial Instruments Inc., Cedar Grove, N.J.) and was proportional to molarity of NaCl.

Analytical procedures. Samples from stock solution (2 mg of dried material per ml of water) were used in analytical procedures. Analyses for nitrogen were performed by Organic Microanalyses Ltd., Montreal. Phosphorus was determined spectrophotometrically (3) with KH_2PO_4 as the standard. The ash content was determined gravimetrically after heating to constant weight at 800°C.

Amino acid composition was determined after hydrolysis in vacuo of 4- to 5-mg samples with 0.5 ml of 6 N HCl containing oxalic acid (1 mg/ml) (20) at 110°C for 24 and 48 h. The hydrolysates were dried in vacuo and dissolved in 1.0 ml of 0.02 N sodium citrate buffer, pH 2.2. The amino acids were analyzed with a Beckman amino acid analyzer model 120C (Beckman Instruments, Inc., Palo Alto, Calif.). Basic amino acids were separated on a column (16.5 by 0.9 cm) (9). After the application of the sample, the space above the resin was filled with pH 3.25 buffer (44), and then the column was developed at pH 5.25. This allowed the separation of lysine from an unknown substance and also improved the resolution of tyrosine/phenylalanine and glucosamine/galactosamine.

Amino sugars were analyzed with the amino acid analyzer after hydrolysis in vacuo with 2 N HCl (36) containing oxalic acid (1 mg/ml) at 105°C for 8, 16, 24, 72, and 96 h. The hydrolysate was analyzed both by the modified short-column procedure and by using the long-column of the amino acid analyzer.

Neutral sugars were determined, after hydrolysis, by gas-liquid chromatography of their trimethylsilyl derivatives. The procedure was based on the method of Lehnhardt and Winzler (28). Samples (1 mg) were hydrolyzed with ion-exchange resin in 0.02 N HCl at 100°C for 24, 48, and 72 h (35). The samples were recovered from the resin, dried by rotary evaporation, treated with 0.5 ml of dimethyl formamide containing 0.1 M 2-hydroxypyridine, and allowed to mutarotate overnight at 40°C (40). The sugars were silylated by shaking for 30 min with 0.5 ml of hexamethyldisilazane and 0.25 ml of trimethylchlorosilane. Samples (2 μl) were injected into a Hewlett-Packard 7610A dual-column gas chromatograph (Hewlett-Packard Co., Mountain View, Calif.) fitted with flame ionization detectors and direct-on-column injection. Flow rates were: He, 60 ml/min; H_2, 35 ml/min; and air, 500 ml/min. Dual copper columns (2.4 m by 6 mm) contained 10% (wt/wt) SE-52 on Diaport S (80 to 100 mesh), and the chromatograph was operated isothermally at 190°C with a flash heater temperature of 260°C and a detector temperature of 270°C. Molar response factors for each sugar relative to mannitol (12) and the percentage composition of each of the sugars at mutarotation equilibrium were determined. Uronic acids were estimated colorimetrically with metaphenylphenol, using glucuronolactone as the standard (7).

The method of O-acetyl determination involved transesterification with sodium methoxide (0.1 M in absolute methanol) at 0°C for 30 min (48). The resulting methyl acetate was estimated by gas chromatography (P. E. Reid, personal communication) in a Hewlett-Packard F&M 402 gas chromatograph fitted with flame ionization detectors.

Infrared spectra were obtained by using approximately 1.5 mg of dry material which was ground with 200 mg of dry KBr, compressed at 18 tons (19.8 metric tons), and scanned in a Unicam SP 200G spectrophotometer (Pye Unicam Ltd., Cambridge, England).

RESULTS

Growth patterns. All strains grew well at 25°C, and exponential phase was established after 36 h. The pH optima for cell growth and subsequent capsule production for all five strains were between pH 6.5 and 7.0 at inoculation, although capsular material and visible capsules did not appear in the medium or on the cells until the pH dropped to below 5.1. The capsule thickness ranged from 1.5 to 4.0 μm.

Virulence tests. Both *C. albidus* and *C. laurentii* failed to kill any of the mice. *C. neoformans* 365-11 killed 50% of the mice after 5 days, and *C. neoformans* 365-16 caused 50% mortality after 18 days.

Electrophoresis of extracellular material. Polyacrylamide electrophoresis patterns of the extracellular materials revealed congruence of periodic acid-Schiff and amido Schwarz bands, indicating that there was only one major glycoprotein. Cellulose acetate electrophoresis revealed a single periodic acid-Schiff and Alcian blue positive band in each preparation. From *C. albidus* this band had a mobility of 0.28 relative to heparin, from *C. laurentii* the value was 0.48, and from *C. neoformans* the value was between 0.31 and 0.34. The viscosity of the material in solution imposed limits upon loading for electrophoresis.

Gel chromatography. The fractionation of extracellular material on a DEAE-Bio-Gel A column produced a single, sharply defined band which eluted at the start of the NaCl gradient. The pattern for *C. neoformans* 365-11 is shown in Fig. 1. Protein and carbohydrate moieties were coincident within this band. This observation, together with the electrophoretic results, suggests that a small protein fraction may be bound to a large acidic carbohydrate moiety. There did not appear to be contamination by other molecular species.

N, P, and ash analyses. The total N in any preparation was between 0.1 and 0.2% by weight. The recovery of N calculated from amino acid analyses was between 40 and 100% of the total N analysis. The total P was between 0.1 and 0.2% except for the *C. neoformans* strains, where the P content was 0.02 to 0.4%. The ash contents varied substantially among preparations but usually were between 0.2 and 0.1%.

Amino acid and amino sugar composition. The commonest amino acids were aspartate/asparagine (Asx), glutamate/glutamine (Glx), serine, and threonine. *C. neoformans* 365-16 had large amounts of proline and differed substantially in the ratios of glycine/alanine, isoleucine/leucine, and tyrosine/phenylalanine from the other strains and species. *C. neoformans* 365-11 bore more resemblance to the non-

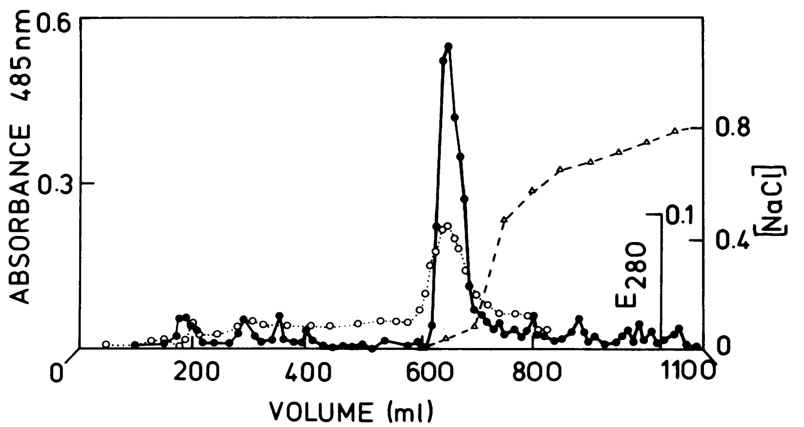

FIG. 1. *Chromatography of extracellular polysaccharide from C. neoformans 365-11 on a DEAE-Bio-Gel A column (37.0 by 2.5 cm). A gradient from 0 to 3.0 M NaCl in 0.02 M pyridine hydrochloride buffer, pH 5.5, was applied after elution with 600 ml of pyridine hydrochloride buffer. Fractions of 10 ml were collected. Symbols: (●) Carbohydrate; (○) protein; (△) NaCl gradient.*

pathogenic species although it had a large amount of Asx. The extracellular material contained lesser amounts of total basic amino acids than did cell walls (0.1 and 0.2%, respectively) and also more serine and threonine (0.7 and 0.1%, respectively). Analysis of all the strains showed a shoulder emerging just ahead of lysine, and *C. albidus* gave a clearly resolved peak before lysine. Spectrophotometric tests for hydroxyproline were all negative. Glucosamine was detected in hydrolysates of all preparations (Table 1), but no galactosamine was detected in the strains examined.

Neutral sugars. Table 2 shows results of neutral sugar analyses. The same four sugars were detected in all strains, although the proportions varied substantially. Mannose was the major constituent in all samples. Total recoveries were approximately 30% higher after resin hydrolysis than after hydrolysis using 2 N trifluoroacetic acid (2, 9).

Uronic acids and *O*-acetyl groups. Table 3 presents results of uronic acid and *O*-acetyl group analyses. The *C. neoformans* strains contained much more uronic acid than did the nonpathogenic species. *O*-acetyl groups were detected only in *C. neoformans*. Strain 365-11, which had a higher 50% lethal dose in our tests, had substantially more *O*-acetylation than strain 365-16. These results indicate that 35% of the total polysaccharide was *O*-acetylated in the extracellular material from *C. neoformans* 365-11. The value for 365-16 was 7%. We did not determine where the *O*-acetyl groups were substituted or in which sugars they occurred.

Complete analysis of the extracellular material. Table 3 summarizes total recovery of the extracellular material components for the four *Cryptococcus* strains. Absolute recoveries ranged from 99.2 to 131.7% by weight. We cannot account for this major discrepancy in absolute recovery. Lipid determinations are not included because gravimetric methods were found to be unreliable on the small amounts available. However, the values obtained were never less than 3% by weight.

TABLE 1. *Amino acids and amino sugars in the extracellular material of Cryptococcus species*

Amino acid	% of total anhydro amino acids and amino sugars recovered[a]			
	C. albidus H1354	*C. laurentii* 371-1	*C. neoformans* 365-11	*C. neoformans* 365-16
Lys	1.8	2.0	2.5	1.6
His	1.6	4.4	1.7	1.0
Arg	2.7	2.5	2.2	1.3
Asx	8.7	11.3	13.4	13.5
Thr	9.9	8.6	10.7	8.7
Ser	10.8	10.0	13.8	15.0
Glx	15.7	13.6	12.9	8.7
Pro	5.5	7.3	4.8	9.7
Gly	7.5	5.5	4.7	3.7
Ala	10.1	10.3	8.5	11.8
Cys	4.4	1.8	0.8	0.0
Val	6.0	4.1	4.8	5.7
Met	0.4	0.6	1.0	0.2
Ile	3.0	3.1	2.9	6.6
Leu	3.7	4.6	4.3	3.1
Tyr	2.6	3.2	3.7	1.3
Phe	2.4	3.3	2.4	6.3
$GlcNH_2$	3.2	4.1	5.1	1.9

[a] For amino acids duplicate samples (4 to 5 mg/ml) were hydrolyzed with 6 N HCl containing oxalic acid (1 mg/ml) for 24, 48, and 72 h. For amino sugars, duplicate samples (4 to 5 mg/ml) were hydrolyzed with 2 N HCl containing oxalic acid (1 mg/ml) for 8, 16, 24, 72, and 96 h.

TABLE 2. *Neutral sugars in the extracellular material of Cryptococcus species*[a]

Sugar	% of total anhydro neutral sugars recovered			
	C. albidus H1354	*C. laurentii* 371-1	*C. neoformans* 365-11	*C. neoformans* 365-16
Xylose	14.4	29.0	15.6	20.8
Mannose	34.2	45.1	69.7	41.9
Galactose	31.3	21.9	11.8	27.3
Glucose	20.1	4.0	2.9	10.1

[a] Hydrolysis and analysis were conducted according to a modification of Lehnhardt and Winzler's (28) procedure (see text).

TABLE 3. *Complete analysis of extracellular material of Cryptococcus species*

Material	Amt in prepn (μg/ml)			
	C. albidus H1354	*C. laurentii* 371-1	*C. neoformans* 365-11	*C. neoformans* 365-16
Polysaccharide				
Anhydro neutral sugar	1,148.5	896.0	1,014.9	990.0
Anhydro amino sugar	3.8	2.1	1.5	0.4
Anhydro uronic[a] acid	56.0	32.2	158.0	112.2
O-acetyl[b]	0.0	0.0	67.1	14.2
Protein				
Anhydro amino acid	103.4	49.0	28.0	20.9
Ash	3.6	11.6	4.5	2.2
Phosphorus	1.3	1.3	0.4	0.2
Total recovery	1,316.6	992.2	1,274.4[c]	1,140.1[c]

[a] Determined spectrophotometrically (7).

[b] Detected as methyl acetates by gas chromatography (see text).

[c] Corrected for H atoms on the sugar molecule which were substituted by *O*-acetyl groups.

Infrared spectroscopy. Material from the four strains showed identical spectral patterns for wave numbers between 2,000 and 4,000 cm^{-1} (Fig. 2). Authentic mannan showed the same absorption maxima at 2,900 and 3,250 to 3,550 cm^{-1} (19). Patterns were also similar between 650 and 1,800 cm^{-1} (Fig. 3), but there were differences in intensity at 1,730, 1,650, 1,550, 1,375, 1,250, and 800 cm^{-1}. Material from *C. laurentii* and a standard for glucosamine hydrochloride lacked the intense peak at 1,730 cm^{-1} caused by carbonyl stretching vibrations. This peak was present in the *C. neoformans* strains. *C. laurentii* and *C. albidus* showed vibrations at 1,550 and 1,620 to 1,635 cm^{-1}. This correlated with larger amounts of amino acids and glucosamine recovered from these strains (Table 3). The signal is attributed to C, O, and NH groups (4). The increased intensity of vibrations at 1,250 cm^{-1} and 1,730 cm^{-1} (Fig. 3) correlated with increased acetylation and uronic acid content (Table 3). A similar slight increase in methyl H at 1,375 cm^{-1} and in carboxylate anion at 1,425 cm^{-1} also confirmed the increase in *O*-acetyl and carboxylic acid present. The more virulent *C. neoformans* 365-11 showed the more intense bands at all of these wave numbers.

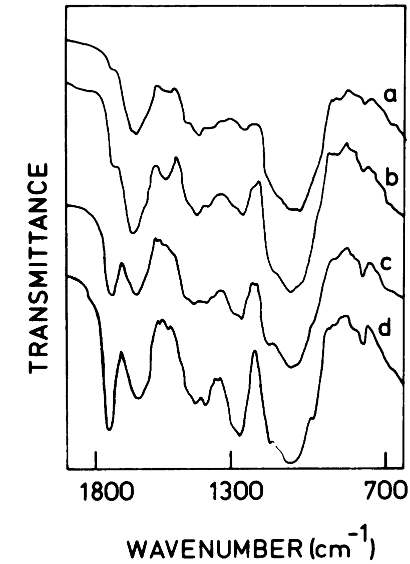

FIG. 3. *Infrared absorption spectra of extracellular material from Cryptococcus species. (a) C. laurentii 371-1; (b) C. albidus H1354; (c) C. neoformans 365-16; (d) C. neoformans 365-11.*

DISCUSSION

Standard culture conditions were adopted for maximal capsule production in LCM. They were not the conditions assumed to occur in vivo, but were selected so that comparative experiments and analytical procedures could be performed on materials produced under identical, and thus comparable, culture conditions. A temperature of 25°C was chosen because the *C. albidus* strain did not grow at 37°C. All strains required thiamine, although we found that a final concentration of 10 μg/ml gave greater capsule production than the 1-μg/ml concentration recommended (30). All strains grew poorly above pH 7.0. This contrasts with a report (8) that maximal capsule size was obtained on solid LCM at pH 7.5 and with the fact that *C. neoformans* must be able to grow at pH 7.34 (spinal fluid) and 7.39 (blood plasma) in vivo. We confirmed other reports (1, 15) that large amounts of heteropolysaccharide were produced by cells inoculated in LCM at an initial pH of 7.0. Recoveries of extracellular material were poor when cells were harvested before they reached stationary phase. We did not determine the cause of the pH drop toward pH 3.0 in this phase. After 5 days of incubation, the *C. neoformans* cultures were much more mucilaginous and slimy than those of the nonpathogens, and the ethanol precipitates from culture fluid were more fibrous. The increased viscosity seemed to be accompanied by increases in *O*-acetyl and uronic acid contents (Table 3).

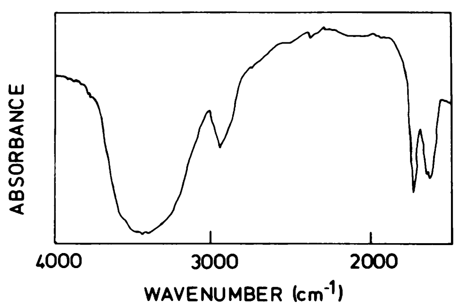

FIG. 2. *Infrared absorption spectrum of extracellular material from C. neoformans 365-11.*

Column chromatography (Fig. 1) and electrophoretic analysis followed by various tests for carbohydrate, protein, and uronic acid indicated that the extracellular material behaved as a single molecular species. Our tests for homogeneity did not reveal the presence of any contaminating materials.

The proportions of mannose, xylose (Table 2), and uronic acid (Table 3) in our preparations were similar to those in other reports (6, 14, 18, 43) but different from results obtained for *C. neoformans* serotype B (5), which was recently reclassified as *C. bacillisporus* (26). The presence of galactose (which we found in all of our preparations) is a subject of conflict. Some workers (13, 39) found two major capsular polysaccharides, only one of which contained galactose. Golubev et al. (16) reported that capsular polysaccharides contained galactose, whereas those from the extracellular material did not. Still others (14) found no difference in the galactose content of adhered and soluble polysaccharides. Our preparations were derived from soluble and easily released capsular materials. Our detection of glucose is the first for this sugar as a component of cryptococcal extracellular polysaccharides.

The major differences between the strains were in uronic acid content, first reported by Kozel and Cazin (24), and in the degree of *O*-acetyl substitution. These differences were clearly shown in the infrared spectra (Fig. 3) in spite of the difficulties in interpretation which often arise because of the additive effects and influences of the absorptions of the different chemical groups present. The peak at 1,250 cm^{-1} is undoubtedly due to C-O stretching vibrations, but the line between the ester function at 1,725 to 1,750 cm^{-1} and the carboxyl at 1,650 to 1,725 cm^{-1} is hard to distinguish, especially if both functions are known to be present. It has been found (17, 46) that the ester function, presumed to be *O*-acetyl, is hydrolytically cleaved under mild alkaline conditions with resulting losses of absorption at 1,725 and 1,250 cm^{-1}. The nonpathogenic species did not contain *O*-acetyl functions, but did have uronic acid. It is thus possible that the vibrations at 1,730 cm^{-1} were due to both acetyl and carboxyl functions. This is consistent with the increase in peak size at this wave number to a maximum for *C. neoformans* 365-11. If the effect is additive, it explains why the difference in peak size between the two strains of *C. neoformans* was not as marked as would have been expected from differences in their *O*-acetyl content alone. None of the strains showed a carboxyl salt peak at 1,600 cm^{-1}. *O*-acetyl groups have been reported from *C. laurentii* grown on low-P medium (42, 44) and from *C. bacillisporus* serotype B (5).

The higher glucosamine content of *C. albidus* and the *C. laurentii* strain (Table 1) is apparent from the spectral enhancement at 1,550 cm^{-1} (Fig. 3). The slight shoulder at 1,730 cm^{-1} on the *C. albidus* spectrum may have been due to *N*-acetylation. Glucosamine has not previously been detected in cryptococcal extracellular polysaccharides or capsules, although other yeasts and both yeast mycelial phases of *Sporothrix schenckii* produced extracellular polysaccharides which contained glucosamine and *N*-acetylglucosamine (21, 45). All of the strains showed peaks at 800 and 900 cm^{-1} representative of α-linked glycan. The vibrations, as well as vibrations at 980 cm^{-1}, were present in the yeast mannan but not in the yeast glucan (4).

This study is the first report of amino acid analyses from cryptococcal extracellular polysaccharide. From 20 to 100 μg of amino acids was recovered per mg of extracellular material for all the strains, and all contained at least 0.1% (by weight) nitrogen (Table 2). Most authors have regarded the N component as a contaminant. They deproteinized by extraction with chloroform-butanol (25) during purification (14, 24, 34). This procedure left about 1.7% (by weight) protein in their preparations, and there may have been more in the original extracellular material because they used 3% chloroform or 1% phenol, the latter of which reduces amino acid recoveries (A. Ross, Ph.D. thesis, University of British Columbia, Vancouver, 1975) to kill the cells before polysaccharide extraction. Threonine, serine, and Glx were the predominant amino acids in *C. albidus*, whereas *C. neoformans* 365-11 had large amounts of Asx, serine, and Glx (Table 1). In *C. neoformans* 365-16, Asx and serine were the predominant amino acids and levels of proline and phenylalanine were higher than in the other strain. The *C. laurentii* had large amounts of Asx and Glx. The discrepancies in N recoveries suggest that there were some losses in amino acids and amino sugars. The presence of large quantities of polysaccharide causes humin production during hydrolysis (20), and it has been shown that some losses of amino acids are due to adsorption to the glass during desiccation (41).

Our use of virulence testing was limited to the confirmation of pathogenic activity in the cultures which were used to inoculate for glycoprotein production. *C. neoformans* strains were pathogenic to the Swiss white mice used in our tests, although 50% lethal dose values differed from those obtained with other strains of mice. Our results support previous reports (17, 29) that reactivity of different serotypes of *C. neoformans* depends to some extent on the degree of acetylation. It is well established (25, 31) that serum opsonizing agents (immunoglobulin G) react with capsular polysaccharide to destroy the pathogenic function (23). Kozel and Cazin (24) found that injection of capsular polysaccharide from a virulent strain of *C. neoformans* did not inhibit phagocytosis in vitro of a nonencapsulated strain or of *C. laurentii*.

C. albidus, the cerebrospinal fluid isolate, was not virulent in our tests. It did not grow at 37°C and was not acetylated. It did have a large amount of galactose (like 365-16) and glucosamine (like 365-11).

Table 3 shows that total recoveries are unreliable measures of success in complete analysis.

Complete (90 to 100%) recovery of cell wall constituents has been achieved (9). The removal of water before analysis proved difficult in that study. The extracellular materials reported here were difficult to dry to constant weight before analysis. We believe that the results reflect failure to dry (heat at 80°C overnight, cool over P_2O_5, and weigh 20 s after removal from desiccator) the sample. The paradox is that different batches of material gave similar results and the error was thus constant. Clearly the matter requires further study. It should be noted that the conclusions reached from this study remain the same if all data are calculated to 100% recovery.

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